Impact of *Pseudomonas graminis* strain CPA-7 on respiration and ethylene production in fresh-cut 'Golden delicious' apple according to the maturity stage and the preservation strategy

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Abstract

The effect of the biocontrol agent (BCA) *Pseudomonas graminis* CPA-7 on the accumulation of CO_2 and ethylene (C_2H_4) in fresh-cut apples at two maturity stages was evaluated in refrigerated conditions. The influence of factors involved in the preservation strategy upon commercial conditions such as the antioxidant (AOX) treatment and the storage system was included in the analysis. Regardless of the maturity stage, the BCA reduced C_2H_4 levels within the MAP atmosphere in AOX-untreated apples wedges, by 29 and 43 % in immature and mature apples, respectively. Nevertheless, the addition of ascorbate as antioxidant counteracted this effect. *In vitro* tests suggested that the reduction of C_2H_4 levels was not associated to the uptake of this molecule by CPA-7. Interestingly, in non-inoculated samples AOX treatment showed contradictory effects on C_2H_4 production in MAP conditions by significantly reducing C_2H_4 levels in immature apples (by 23 %) while increasing it in mature ones (by 40 %). Similarly, CPA-7 had opposite effects on the CO_2 accumulation pattern depending on the storage system or the fruit maturity stage. In this sense, CPA-7 was associated to higher fruit respiratory activity at advanced maturity stages yet

without inducing the fruit fermentative metabolism or altering fruit quality during a week of refrigerated storage. Overall, these results show that CPA-7 may contribute to the overall maintenance of the microbiological and physicochemical quality of fresh-cut apple by modulating the fruit ethylene production and/or respiration.

Key words:

Antagonist, fresh-cut fruit physiology, passive modified atmosphere packaging, MAP

1. Introduction

The effect of the application of antagonists on fresh-cut produce in commercial conditions is influenced by internal factors like the type and the maturity stage of the commodity and external factors such as temperature, preservative treatments as well as oxygen and carbon dioxide concentrations within packages. From the physiological stand, processed products essentially behave as wounded tissues where the disruption of cell compartmentalization lead to the mixture of cellular components with an increase of enzymatic and respiratory activities as well as an elevated production of ethylene (C_2H_4) (Hodges and Toivonen, 2008; Mahajan et al., 2014). C_2H_4 is also indeed, the main hormone controlling ripening in clicmateric fruit (Reid et al., 1973) and its biosynthesis involves the transformation S-adenosylmethionine into the precursor 1-aminocyclopropane (ACC) mediated by the enzyme ACC synthase (ACS). ACC is later converted to ethylene by ACC oxidase (ACO) (Yang and Hoffman, 1984). In apple, as a fruit with a climacteric behavior, the regulation of these two steps is auto-inhibitory during fruit development prior to ripening and auto-stimulatory at the onset of ripening (Tatsuki, Endo, & Ohkawa, 2007; Wang et al., 2009; Lelièvre, Latché, Jones, Bouzayen, & Pech, 1997). Processing implies the mechanical injury of fruit tissues which induces the activity and synthesis of ACS leading to the formation of “wound ethylene” whose accumulation may be enough to activate the clicmateric phase depending of the size and permeability of packages (Lamikanra, Imam, & Ukuku, 2005; Yu & Yang, 1980). However, the low availability of ACO in pre-climacteric apples is a

limiting factor regulating ethylene production upon cutting due to the reduced capability for the conversion of ACC into ethylene (Lara and Vendrell, 2000). In the post-clicmateric stage the capacity for ethylene production is also reduced and response to wounding is more limited than in the clicmateric stage (Abeles et al., 1993). Therefore, the physiological stage of the commodity constitutes a key factor that should be taken into account in the production of fresh-cut fruit products (Toivonen and Dell, 2002).

Respiration also shows a biphasic rise during the development of clicmateric commodities, the first one early in development and the second one during ripening or senescence. The second peak usually precedes the autocatalytic ethylene synthesis stage (Fonseca et al., 2002). Moreover, respiration is also induced by cutting due to the loss of compartmentalization of the enzymes involved in the respiration pathways and its substrates, and the activation of key regulatory steps of glycolysis and the tricarboxylic acid cycle (Rolle and Chism, 1987). The mechanical injury of cell membranes also activates the enzymatic degradation of its lipid components, with the formation of long-chain fatty acids whose α-oxidation also causes a rise in respiration (Rolle and Chism, 1987). It is also well stablished that 'wound ethylene' induces fruit respiration (Yu and Yang, 1980). Furthermore, an increase in CO_2 production also occurs in fresh-cut tissues due to the activation of cell repair processes, not only for obtaining energy but for the synthesis of replacement structural compounds (Gomez-Lopez, 2012). The accelerated oxidative breakage of organic substrates and the loss of structure of membranes entailed by the above mentioned processes are detrimental to the nutritional properties and the overall quality of fresh-cut fruit (Soliva-Fortuny & Martín-Belloso, 2003).

To reduce both respiration and ethylene production several methods comprising chilling conditions and modified atmosphere packaging (MAP) are amongst the most currently used in the fresh-cut produce industry (Rupasinghe and Yu, 2013). The addition of biocontrol agents (BCA) such as *Pseudomonas* spp. is another method that

could contribute to modulate ethylene levels thereby extending the shelf-life of fresh ready-to-eat products. Mechanisms for the modulation of plant ethylene metabolism by *Pseudomonas* spp. have been already documented and may imply both its exacerbation or its reduction (Fatima & Anjum, 2017; Glick, 2014; Hase et al., 2003). To accomplish the first mentioned effect, Pseudomonads enhance the plant capacity to transform the precursor ACC into ethylene, inducing the expression of C_2H_4-responsive genes (Hase et al., 2003). Consequently, systemic induced resistance (ISR) is triggered or primed allowing plants to respond better to a subsequent infection by a broad spectrum of pathogens (Van Wees et al., 1997). The C_2H_4-reducing effect has been observed in plants upon treatment with pseudomonads with ACC deaminase (ACD) activity (Hernández-León et al., 2015; Singh et al., 2015). ACD cleaves ACC into ammonia and α-ketobutyrate (Honma and Shimomura, 1978) lowering the amount of available ACC and therefore limiting ethylene synthesis (Glick, 2014). As a consequence of this process pseudomonads can delay ripening and senescence, promote growth, prime resistance mechanisms and alleviate deleterious ethylene-mediated plant stresses (Eckert et al., 2014; Glick, 2005; Wang, Knill, Glick, & Défago, 2000). Belonging to this bacterial group is *Pseudomonas graminis* CPA-7, an apple epiphyte biopreservative strain which controls foodborne pathogens such as *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enterica* on fresh-cut fruit (Alegre et al., 2013a, 2013b; Abadias et al., 2014; Collazo et al., 2017) and modulate oxidative metabolism in fresh-cut apple (Collazo et al., 2018). In an attempt to clarify its mode of action we also investigated the possibility for CPA-7 to modulate the ethylene metabolism in fresh-cut apple thereby influencing the defense response and/or the senescence of this fruit. With this in mind, we monitored the effect of the antagonist in fresh-cut apples as affected by several factors involved in production and commercial conditions (antioxidant treatment, packaging headspace gas composition, and the maturity stage of the commodity). In addition, to assess the effect of CPA-7 metabolic

activity in supplying exogenous ethylene or metabolizing the produced by the fruit, the ability of the antagonist to either produce or consume C_2H_4 was tested *in vitro*.

2. Materials and methods

2.1 Antagonist inoculum preparation

Pseudomonas graminis strain CPA-7 (Alegre et al., 2013b) inoculum was obtained by centrifugation of 50 mL of an overnight culture in tryptone soy broth (TSB, Biokar, Beauveais, France) at 25 ºC in agitation (Collazo et al., 2017). The concentration of the solution was checked by viable plate count of appropriate ten-fold dilutions in saline peptone (8.5 g L^{-1} NaCl, 1 g L^{-1} peptone) onto TSA plates after incubation at 30 °C for 48 h.

2.2 Fruit processing

Apples (*Malus domestica* Borkh. cv. 'Golden delicious') used in this study were grown in local farms (Lleida, Catalonia, Spain) and collected in august, 2017 at two maturity stages (with a week of difference between harvests). Prior to experimental assays, apples were washed with running tap water, surface disinfected with 700 mL L^{-1} ethanol and either stored as such or processed (peeled with an electric fruit peeler and cut into eight wedges with a handheld corer/slicer). Wedges were kept in chilled (5 °C) chlorinated tap water (pH 6) until treatment and/or packaging.

2.3 *In vitro* analysis of ethylene production or consumption by CPA-7

2.3.1 Preparation and inoculation of liquid culture media

In vitro assays were performed in order to evaluate the putative ethylene production or consumption by CPA-7 in a culture medium with a similar composition to the fruit but discarding the changes due to the apple's native microbiota. For that, analysis glass tubes containing 10 mL of apple juice were inoculated with CPA-7 to a concentration of 10^5 CFU mL^{-1}. Aliquots of TSB were prepared, inoculated and analyzed in the same way, as a control treatment. For juices preparation, apple wedges were previously dipped in 6% NatureSeal® AS1 solution (AS1, AgriCoat Ltd., Great Shefford, UK), a

calcium ascorbate-based product or in cold deionized water for 2 min in agitation (15.7 rad s^{-1}) in a tabletop orbital shaker (Unimax 1010, Heidolph, Germany). Then, juices were obtained in a commercial blender, subsequently filtered through cloth gauzes and either adjusted to pH 6.5 with 1 mmol L^{-1} NaOH or sterilized as such at 215 ºC for 5 min and stored at 5 ºC until use. Non-inoculated aliquots of each culture medium were also prepared and used as controls. Inoculated and non-inoculates tubes were stored in aerobic conditions at 5 ºC or 25 ºC in agitation for 7 d. *In vitro* assays were repeated twice and included three replicates per treatment.

2.3.2 *In vitro microbial dynamics*

CPA-7 population dynamics in each culture medium was tracked by viable plate count on TSA at 0, 1, 3, 6, and 7 d post-inoculation, as described in section 2.1.

2.3.3 *In vitro* CO_2 accumulation pattern

The headspace gas composition (percentages of O_2 and CO_2) of each culture tube was measured at 0, 1, 3, 6, and 7 d post-inoculation using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark). Before each measurement tubes were hermetically closed for 12 h. CO_2 accumulation was expressed in mg mL^{-1} liquid culture medium.

2.3.4 *In vitro* ethylene accumulation

The ethylene accumulation patterns of cultures tubes previously sealed for 12 h were determined at 0, 1 and 3, 6 and 7 d post-inoculation. For that,1 mL of gas sample was withdrawn daily from each jar or tray with a syringe and injected into a gas chromatograph (Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1,Tecknokroma, Barcelona, Spain). The injector and detector were kept at 180 °C and 280 °C, respectively. Quantification was carried out by comparing the gas chromatography signal of the samples to that of a 21 µL L^{-1} C_2H_4 standard (Carburos metálicos SL, Aragón, Spain). Ethylene accumulation within the storage atmosphere of the tubes was expressed as µL mL^{-1} culture medium. Putative ethylene degradation by CPA-7 was assessed by

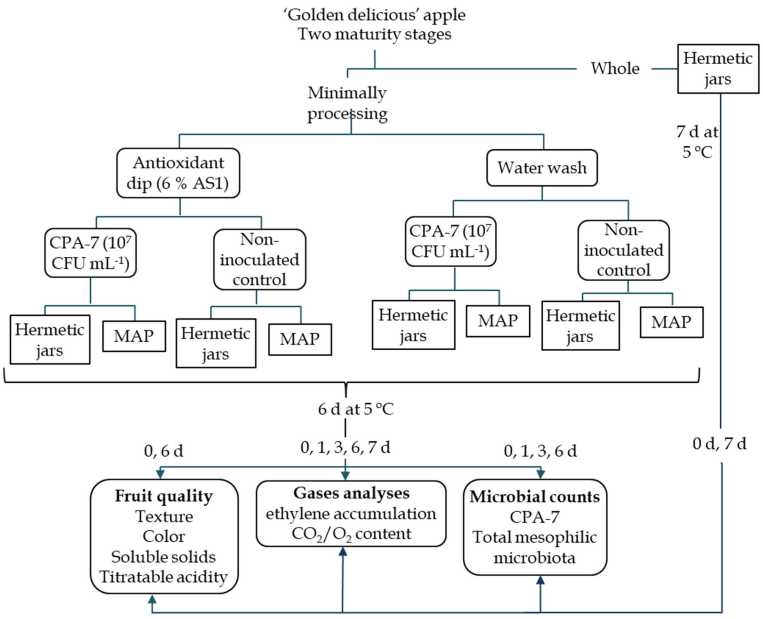
tracking the reduction of 1 mL of 21 µL L^{-1} C_2H_4 standard injected with a syringe in hermetically sealed tubes (containing 10 mL of either apple juice or TSB medium and 12 mL of headspace).

2.4 *In vivo* analysis of ethylene and CO_2 accumulation patterns in CPA-7-inoculated fresh-cut apples

2.4.1 Fruit treatment and packaging

The experimental setup of *in vivo* tests is shown in Figure 1. For dip inoculation of apple wedges, CPA-7 suspensions at a concentration of 10^7 CFU mL^{-1} were prepared in cold deionized water (4 ºC) or in cold 60 g L^{-1} AS1 antioxidant aqueous solution. Fruit wedges were dipped in the bacterial suspensions or in non-inoculated water or antioxidant solution as controls, at a ratio of 1:2 (weight of fruit: volume of solution) as described in section 2.3.1.

After the drainage of the excess of water, the treated apple wedges were packaged in two storage systems; hermetic jars (static system) and commercial trays (MAP). For the static system, 1 kg of intact apples was stored in 3.4 L jars and 500 g of apples wedges was stored in 1.7 L jars. Jars were equipped with a silicon septum for sampling the gas of the headspace. For MAP, 120 g of processed fruit were placed in 400 mL polyethylene terephthalate ShelfMaster™ Pronto™ trays (PlusPack, Denmark) and thermosealed with 181.7 cm^2 of a 3-holed (60 - 80 µm diameter, 75 mm spacing) multilayered microperforated film (polyester anti-fog film, OALF (14 µm of thickness) + oriented polypropylene, OPP (20 µm of thickness) (PDS Group, Murcia, Spain) to achieve passive modified atmosphere. Trays and jars were stored statically at 5 °C in darkness. Each tray/jar was considered as a replicate and three replicates per treatment and sampling time were included.



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188 Figure 1. Experimental workflow of *in vivo* trials

189 ### *2.4.2 In vivo* microbial dynamics

190 CPA-7 as well as total mesophilic microorganisms' population dynamics on apple
191 wedges stored in trays were analyzed at 0, 1, 3 and 6 d post-inoculation. For this, 10 g
192 of apple from each tray was homogenized in 90 mL buffered peptone water (BPW,
193 Biokar, Beauvais, France) and analyzed by viable cells count on TSA plates for CPA-7
194 and on plate count agar plates (PCA, Biokar, Beauvais, France) for total mesophilic
195 microbiota. Plates were incubated at 30 ºC for 48 h. In the same way, microbial
196 populations in jars were determined at 0 and 7 d post-inoculation. Microbiological data
197 were expressed as colony forming units per gram of fresh weight of fruit (CFU g^{-1} FW)
198 and transformed to $\log_{10}$ for subsequent statistical analyses.

199 ### *2.4.3 In vivo* CO_2 accumulation pattern

200 The headspace gas composition (percentages of O_2 and CO_2) of each replicate, stored
201 in the static system (jars) or in MAP (trays), was measured at 7 h post-inoculation and
202 then at 1, 2, 3, 6, and 7 d as described in section 2.3.3. CO_2 accumulation was
203 expressed relative to the fresh weight of fruit (mg kg^{-1}).

2.4.4 In vivo ethylene accumulation pattern

To determine *in vivo* ethylene accumulation samples were taken from trays and jars at 0, 1, 3, 6, and 7 d post-treatment. Ethylene accumulation within the storage atmosphere was expressed as μL kg^{-1} fresh weight fruit.

2.2.5 Fruit quality parameters

Texture of whole and processed apples as well as color, pH, soluble solids and titratable acidity of apple wedges were determined as described elsewhere (Alegre et al., 2013a). Quality parameters were measured initially and at the end of storage in the case of whole apples and wedges stored in jars while in processed apples stored in MAP they were measured at 0, 1, 3 and 6 d post-treatment. Texture and pH were measured in five wedges per replicate per treatment at each sampling time. Two measures of color, one per side, were performed on five wedges per replicate per sampling time. Soluble solids and titratable acidity of each replicate were measured initially and at the end of storage regardless of the storage system. Low values of CIE color parameter L* and high values of a* were considered as indicators of surface browning intensity (Sapers and Douglas, 1987). The concentration of soluble solids at 20 °C was expressed as mass fraction (%). Titratable acidity was measured in 10 mL of pulp and was expressed as malic acid content (g L^{-1} juice).

2.5 Statistical analysis

Data were analyzed using the general linear model procedure to determine the treatment and interaction effects, with the statistical software JMP (version 11 SAS Institute Inc., NC, USA). All data were verified for normal distribution and homoscedasticity of residues. Results were schematically represented as means ± standard deviation. Means were compared by analysis of variance (ANOVA) and separated by Tukey's test ($P < 0.05$).

3 Results and discussion

3.1 In vitro analysis of microbial dynamics and ethylene production or consumption by CPA-7

To test the ability of CPA-7 to growth and produce or consume ethylene in sterile apple juice, microbial populations and gas headspace composition were measured during 7 days. The same analyses were performed in TSB, a synthetic media usually used for CPA-7 culture in the laboratory, as a control treatment (Abadias et al., 2014; Alegre et al., 2013a). Results showed that CPA-7 initial populations (10^5 CFU mL^{-1}) increased by 1 log_{10} in TSB after 24 h at 5 ºC and afterwards they remained stable up to day 6 (Fig. 2).

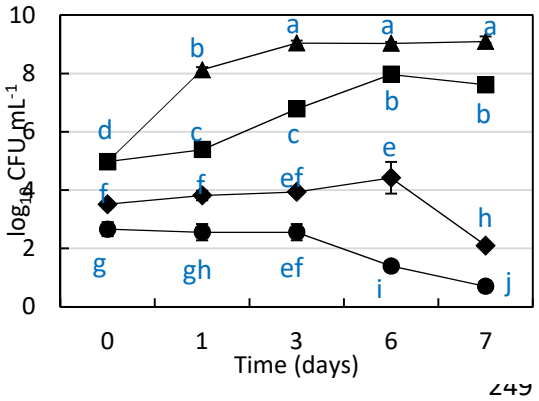


Figure 2. CPA-7 in vitro population dynamics in different growth conditions: apple juice pH 6.5, at 25 ºC (▲), apple juice pH 6.5, at 5 ºC (■), TSB medium at 5 ºC (◆), and apple juice pH 4.5, at 5 ºC (●). Symbols represent means and error bars represent standard deviation of the mean (n = 6). Different letters represent significant differences among treatments according to analysis of variances (ANOVA) and Tukey's test with a 95 % confidence ($p < 0.0001$).

No growth was observed in AOX-untreated apple juice pH 4.5 but instead, the population gradually decreased to levels close to detection limit (5 CFU mL^{-1}), thus the gas analysis of these samples was stopped after day 3. In accordance with this result, previous studies shown that CPA-7 failed to growth in synthetic liquid media adjusted with different organic acids to pH ranging from 4.5 to 5 (Iglesias, 2017). CPA-7 also showed limited growth in melon juices compared to fruit pieces, which is a fruit with pH close to neutrality but rich in citric acid (Collazo et al., 2017) . Therefore, the subsequent analyses were performed in apple juices with pH adjusted to 6.5. Results showed that CPA-7 populations quickly increased (by 3 log_{10} in the first 24 h) in apple juice pH 6.5 incubated at 25 ºC reaching the stationary phase (9 ± 0.1 log_{10} CFU mL^{-1}) before day 3 as expected for the optimal growth temperature (Alegre et al., 2013b).

When incubated at 5 ºC CPA-7 populations in apple juice pH 6.5increased slower than at 25 ºC, reaching the stationary phase on day 6 and attaining levels 2 log_{10} higher than when grown in TSB (pH 7.3). In general results showed that apple juice pH 6.5 a suitable culture medium for CPA-7, showing similar population dynamics to that observed in fresh-cut melon (pH 6.4) incubated at a temperature within the optimal range (20 ºC) (Abadias et al., 2014).

CO_2 production was close to cero during the whole evaluated period in the samples grown in TSB as well as in apple juice pH 4.5 (data not shown), which was in accordance with the lower growth observed in those culture media compared to apple juice pH 6.5. Likewise, greater CO_2 levels correlated with the populations dynamics observed in AOX-treated apple juices (pH 6.5) stored at 5 ºC or 25 ºC, where the CO_2 production remained stable around 0.04 ± 0.04 mg L^{-1} h^{-1} or increased from 1.5 ± 0.4 to 2.9 ± 0.9 mg L^{-1} h^{-1}, respectively.

Regardless of the temperature of storage no ethylene production was observed for CPA-7 whether it was grown in TSB or in apple juices (pH 6.5 or pH 4.5). In the same way, no differences in ethylene accumulation or in the antagonist population levels were detected in the inoculated samples after supplementation with exogenous C_2H_4 compared to the non-supplemented control at any of the analyzed sampling points in any of the culture media or incubation temperatures assayed (data not shown). Overall, results obtained from *in vitro* assays showed that although CPA-7 is able to use the nutrients present in the apple-based food matrix tested to grow, it is unable to produce or consume ethylene in the conditions tested.

3.2 In vivo analysis of ethylene and CO_2 accumulation patterns in CPA-7-inoculated fresh-cut apples

3.2.1 In vivo microbial population dynamics

In MAP-stored apples CPA-7 populations were initially 5.53 ± 0.04 and 5.90 ± 0.04 log_{10} CFU g^{-1} FW in samples from the first (H1) and the second harvest (H2),

288 respectively. Afterwards, in samples upon the antioxidant effect, CPA-7 populations
289 showed a slower growth and reached lower levels than in AOX-untreated samples (Fig.
290 3 A). These results agreed with the those observed for this bacteria when grown on
291 fresh-cut pear upon another antioxidant treatment but in similar storage conditions
292 (Iglesias et al., 2018). Conversely, in apples from the second harvest no differences
293 were observed in AOX-treated and untreated samples during the whole storage period
294 (Fig. 3 B). Likewise, Alegre et al. (2013a) selected the AS1 antioxidant treatment for
295 commercial assays with CPA-7 in 'Golden Delicious' fresh-cut apple wedges as they
296 observed no differences in growth between AS1-treated or untreated samples
297 incubated at 10 ºC during 2 d. Furthermore, they observed increases by 2 $\log_{10}$ AS1-
298 treated samples after 7d of storage at 10 ºC. Discrepancies in the effect of antioxidant
299 treatments on CPA-7 growth between that study and ours might be related to the
300 maturity stage of the commodity used for the different experiments. However, CPA-7
301 grew more in H2 (by 1.2 ± 0.2 $\log_{10}$) than in H1 apples (by 0.8 ± 0.1 $\log_{10}$) regardless of
302 the antioxidant application. On the other hand, when stored in jars, CPA-7 populations
303 on apple wedges showed similar dynamics regardless of the harvest date or the
304 antioxidant treatment (Fig. 3 C and D).

305 Mesophilic bacteria populations were initially at the same levels in samples from both
306 harvest dates (2.3 ± 0.2 $\log_{10}$ CFU g^{-1} FW). As observed for CPA-7, mesophilic bacteria
307 grew more in the AOX-untreated H1 apples (by 1.9 ± 0.4 $\log_{10}$) than in the rest of the
308 samples (by 0.7 ± 0.1 $\log_{10}$) (data not shown). Those results confirmed the inhibitory
309 effect that antioxidant agents have on microbial growth as previously observed in
310 several studies performed with fresh-cut fruit treated with different antioxidant
311 compounds and mixtures (Iglesias et al., 2018).

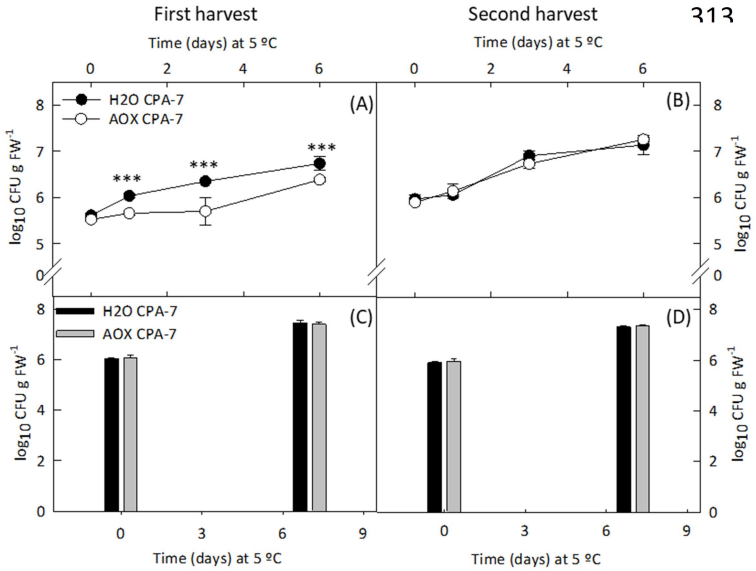


Figure 3. CPA-7 population dynamics on antioxidant-treated (AOX) or untreated (H_2O) fresh-cut 'Golden Delicious' apple wedges from the first (left column) and the second harvest (right column) dates during storage in trays (MAP conditions) (A-B) or in hermetically sealed jars (C-D) at 5 ºC. Symbols represent means and error bars represent standard deviation of the mean (n = 6). Asterisks represent significant differences among treatments according to analysis of variances (ANOVA) and Tukey's test with a 95 % confidence (*** for $p < 0.0001$).

3.2.2 In vivo CO_2 accumulation pattern

The headspace gas composition of packages was highly influenced by the permeability of the storage system and the physiological stage of the fruit (Supplementary Table 1). As expected, apples from the second harvest (H2) showed higher respiration rates than those from the first one (H1) thereby depleting faster O_2 levels. This led to anoxic conditions (0.4 ± 0.3 %) by day 7 of storage in the hermetic system in H2 inoculated samples while in H1 apples O_2 levels only decreased to 12 ± 1 % in the same period. In the permeable system a stable O_2 concentration ranging from 15 to 17 % was maintained throughout storage.

Like ethylene production, respiration is activated both during ripening and upon wounding-stress thus, during the processing of fresh-cut produce (Saltveit, 2016). Accordingly, our results showed an initial rise in CO_2 production by 40 % in processed apples compared to intact apples in both maturity stages (Fig. 4 G-H). The magnitude of the wound-response in the respiration rate was significantly higher in mature than in immature apples exceeding by 2.5-fold and 1.4-fold, respectively, the respiration rate of whole apples. The exacerbation of respiration rate in fresh-cut apple slices by 2 - 3 times compared to whole fruit had previously been reported (Lakakul, Beaudry, & Hernandez, 1999). The increased respiration in minimally processed fruit is mostly due

to a physiological response to wounding which is tightly linked to the maturity stage, since the removal of the peel barely reduce the resistance to O_2 diffusion in apples (Fonseca et al., 2002). In general, we observed that respiration rate reached the highest values immediately after processing and subsequently declined throughout storage regardless of the combination of factors analyzed.

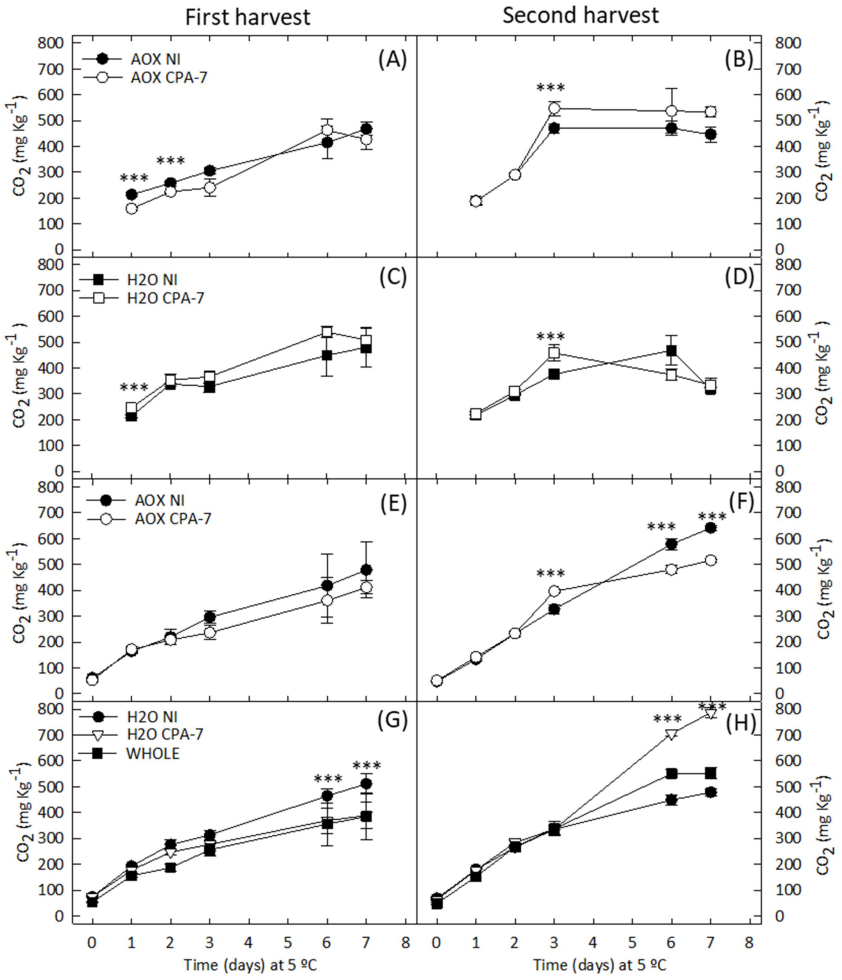


Figure 4. CO_2 accumulation patterns in CPA-7-inoculated and non-inoculated (NI) fresh-cut 'Golden delicious' apple wedges from the first (left column) and the second harvest (right column) dates when stored in MAP (A-D) and in hermetic jars (E-H). Graphs A-B and E-F: apples treated with the antioxidant (AOX); graphs C-D and G-H: AOX-untreated samples. Symbols represent means of three biological replicates and error bars represent standard deviations. Asterisks represent significant differences according to an analysis of variances ANOVA and Tukey´s test with a 95 % confidence (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$). Asterisks below the lines represent differences only between whole and processed apples, regardless of the inoculation with CPA-7.

In MAP storage, CO_2 accumulation in H1 AOX-untreated samples showed an increase by 16 % in the presence of CPA-7 compared to non-inoculated control, 24 h post-

363 processing (Fig. 4 C). The addition of the antioxidant reversed this behavior, being CO_2
364 production in inoculated samples up to 25 % lower than the control during the first two
365 days in MAP conditions (Fig. 4 A). In H2 samples differences in CO_2 accumulation
366 pattern were only observed on the 3rd day of MAP storage being remarkably higher in
367 response to the antagonist than in the control whether they were treated (by 16 %) or
368 not (by 22 %) with the antioxidant. This could be associated to the clicmateric peak of
369 the product as it was correlated with a rise in ethylene production (Lelièvre et al.,
370 1997). According to this result, CPA-7 may enhance the clicmateric peak in processed
371 apples at more advanced maturity stages in agreement with previous findings showing
372 that once the autocatalytic phase of ripening has begun the effectiveness of
373 preservative methods is considerably reduced and shelf-life is shortened (Rojas-Graü
374 et al., 2007; Soliva-Fortuny, Oms-Oliu, & Martìn-Belloso, 2002). Previous experiments
375 performed with fresh-cut 'Fuji' apple slices showed that according to storage
376 conditions, reducing agents such as ascorbic acid influence other physiological
377 processes in addition of preventing oxidation (Gil et al., 1998). In that study, ascorbic
378 acid dips reduced respiration rate as well as ethylene production in 'Fuji' apples stored
379 in air (21% O_2, 0% CO_2) while increased respiration in MAP- stored (0% O_2, 0% CO_2,
380 100% N_2) apple slices (Gil et al., 1998).

381 Contrastingly in the hermetic system (jars), no differences were observed between
382 inoculated and non-inoculated apples during the first two days of storage regardless of
383 the addition of the antioxidant or the harvest date. However, contradictory effects of
384 CPA-7 on CO_2 accumulation according to the maturity stage and the treatment with the
385 antioxidant were observed in the hermetic system from the 3rd day of storage even
386 when population levels were not influenced by the mentioned factors. For instance a
387 decrease (by 22 %; Fig. 4 G) or increase (by 67 %; Fig. 4 H) in CO_2 accumulation was
388 observed in CPA-7-inoculated AOX-untreated samples from H1 and H2, respectively,
389 compared to non-inoculated controls. The impact of CPA-7 on CO_2 accumulation in the

hermetic system was either eliminated (no differences in H1 samples) or inversed (higher by 22 % than the H2 control) upon the antioxidant treatment in both maturity stages (Fig. 4E-F).

The influence of external factors such as temperature and the gas headspace composition (O_2 and CO_2) of the packages on the respiration of fresh-cut commodities is generally recognized and thus it has been included in several models for the design of MAP technologies (Fagundes, Carciofi, & Monteiro, 2013; Lakakul et al., 1999). However the combination of these factors and biological or chemical preservatives has not been so well studied. Investigation on this matter is needed for developing models to predict the shelf-life of fresh-cut produce in commercial conditions.

3.2.3 In vivo ethylene accumulation pattern

The initial ethylene production of intact apples from the second harvest date (H2) was 3.7-fold higher than those from the first one (H1) evidencing their more advanced physiological stage (Lelièvre et al., 1997), thus they will be henceforth referred as mature and immature apples, respectively. In the same way, C_2H_4 production in intact H2 apples remained 3-fold higher than processed ones during the whole storage while it showed no differences in H1 fruit (Fig 5G-H) suggesting the pre-clicmateric stage of the fruit from the earliest harvest (Chaves and de Mello-Farias, 2006; Oetiker and Yang, 1995). The immediate effect of processing in C_2H_4 production was highly influenced by the maturity stage, showing a reduction in mature apples wedges compared to intact fruit and not differences in immature fruit. Contradictory effects of cutting on ethylene production, as previously observed in several clicmateric commodities, have been explained by differences in the physiological stage of the fruit and linked to differential expression patterns of the genes involved in the ethylene metabolism, before, during and after, the clicmateric phase (Bapat et al., 2010; Toivonen & Dell, 2002; Vilanova et al., 2017).

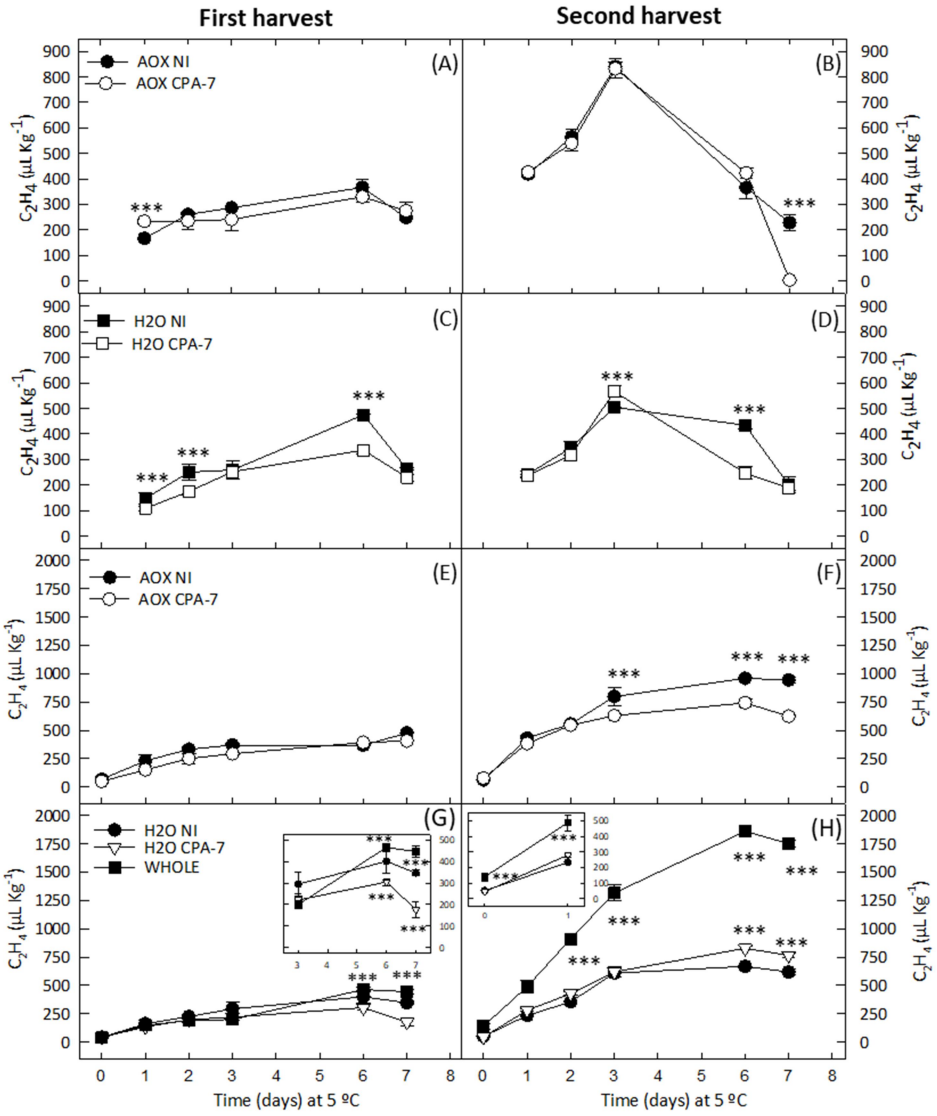


Figure 5. Ethylene accumulation in CPA-7-inoculated and non-inoculated (NI) fresh-cut 'Golden delicious' apples from the first (left column) and the second harvest (right column) dates when stored in MAP (A-D) or in hermetic jars (E-H). Graphs A-B and E-F: apples treated with the antioxidant (AOX); graphs C-D and G-H: AOX-untreated samples. Inserts in graphs G and H are plots of the same data in a smaller scale. Symbols represent means of three biological replicates and error bars represent standard deviations. Asterisks represent significant differences according to an analysis of variances ANOVA and a Tukey´s test with a 95 % confidence (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$).

The impact of the application of CPA-7 on fresh-cut fruit ethylene metabolism cannot be separated from that of other factors such as the gas internal composition of packages, the maturity stage and the preservative chemical treatment which has previously shown to have significant influence on the physiology of this kind of products (Rojas-Graü et al., 2007). In MAP storage, the ethylene accumulation pattern in processed AOX-untreated apples was different according to the maturity stage (Fig. 5,

C-D) showing a peak on day 6 in H1 apples and on day 3 in H2 ones. Similarly, during the first days of storage, differential effects of the application of CPA-7 on the ethylene accumulation within the MAP atmosphere were observed in processed AOX-untreated apples, causing reduced C_2H_4 levels (by 30 %) in immature apples on day 2 while it enhanced them by 11 % in mature apples on day 3. However, as time passed, CPA-7 was associated to a reduction in C_2H_4 accumulation by about 29 % and 43 % in H1 and H2 apples, respectively. This may have in turn contributed to a reduction of the ethylene-mediated fruit senescence (Czarny et al., 2006) and hence, partially explain the improved quality of CPA-7 inoculated apples.

The antioxidant treatment effectively reduced C_2H_4 levels in H1 apples upon MAP conditions during the whole storage while markedly enhanced it in H2 apples (Fig. 5A-B). In general, CPA-7 effect on the C_2H_4 levels within MAP packages was suppressed by the antioxidant, except for the increase by 29 % during the first 24 h post-processing in H1 apples. Likewise, no differences were observed regarding C_2H_4 accumulation within the MAP atmosphere in mature apples until day 6 when it dropped by 98 % in the presence of CPA-7.

In our trials, the reduction of C_2H_4 levels cannot be explained by the consumption of ethylene by the antagonist since the levels of this molecule remained invariable after its supplementation *in vitro,* both in TSB medium and in apples juices. Alternatively, the reducing effect of this bacterium on the fruit ethylene production could be associated to 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase activity. This hypothesis is suggested by the presence in the bacterial genome of a gene fragment encoding a protein (Collazo et al., unpublished results) that shares 98 % homology with the ACC-deaminase (ACD) enzyme (GenBank Acc. No. WP_065986140.1) belonging to *P. graminis* strain P 294/08 (Behrendt et al., 1999). However, further analysis should be performed in order to demonstrate the functionality of this gene in CPA-7. Following this thought, no remarkable reduction of C_2H_4 can be atributed to ACD activity soon

457 after processing since the initial response to wounding has been suggested to deplete
458 the existing pool of ACC available within plant cell through the rapid action of ACC
459 oxidase (ACO), which has greater affinity for ACC than ACD (Glick, 2014). However,
460 ACD activity could explain the reduction of the second ethylene peak, probably
461 associated to *de novo* production of ACC, that was observed in CPA-7-inoculated
462 samples after 6 d of storage. The decrease in C_2H_4 levels at that sampling point was
463 observed in both storage systems (MAP and hermetic) when O_2 concentration was
464 similar (14 – 16 %).

465 In the hermetic system, changes in the accumulation of ethylene in CPA-7-inoculated
466 AOX-untreated apple wedges were only observed at the end of storage and they
467 showed to be highly influenced by the maturity stage (Fig 5, G-H). In H1 apples CPA-7
468 reduced the C_2H_4 accumulation pattern by 24 and 49 % compared to the non-
469 inoculated control after 6 and 7 days of storage, respectively (Fig 5G). In H2 apples
470 inoculation with CPA-7 triggered higher C_2H_4 levels (by up to 19 %) after 6 d of storage
471 (Fig. 5H). The CPA-7 effect was either annulled (no differences in H1 apples) or
472 reversed (decreased by 34 % at day 7 in H2 apples) upon antioxidant treatment (Fig. 5
473 E-F). Similarly, a reduction of C_2H_4 levels in wounded clicmateric melon inoculated with
474 the biological control agent *Bacillus subtilis* EXWB1 was also observed by Wang et al.
475 (2010) after 4 d of storage in hermetically sealed containers at 24 ºC, which correlated
476 with a subsequent increase in the fruit defense response to fungal decay. In addition to
477 the maturity stage, the differences in the C_2H_4 accumulation patterns observed at the
478 end of storage in the hermetic system could also be influenced by O_2 availability, which
479 varied from 12 – 13 % for immature apples to 0.4 – 1.2 % for mature ones. However,
480 reduced C_2H_4 accumulation in respect of the control was observed in mature apples
481 stored in the hermetic system (2.9 – 1.7 % of O_2, 14 % CO_2) when treated with the
482 antioxidant. O_2 is a key factor in the regulation of *AcdS* gene, which encodes ACD
483 (Singh et al., 2015) but limited information is available regarding the influence of

antioxidants in this process. Results showed that antioxidant (AOX) dipping of mature apples enhanced the accumulation of C_2H_4 both in the hermetic and the MAP storage system, regardless of the inoculation with the antagonist. Ascorbic acid-based antioxidant dipping has previously shown to increase ethylene production in fresh-cut Fuji apples upon refrigerated conditions at a different timing and extent according to oxygen availability and ripening stage, showing higher ethylene accumulation in MAP than air conditions and in ripe than in unripe fruit (Rojas-Graü et al., 2007).

3.2.4 Fruit quality parameters

The initial firmness of whole apples was similar regardless of the moment of harvest (67 ± 3 N) but it declined throughout storage in more extent in apples from the second harvest (H2) than in those from the first one (H1): by 45 and 26 %, respectively. In general, no significant differences in firmness were observed among treatments or throughout storage in MAP or in jars, with values ranging from 13 ± 2 and 10 ± 1 N (data not shown). No significant reduction of texture was either observed upon inoculation with CPA-7 and MAP storage for 5 to 10 d at 5 or 10 ºC in previous experiments performed in fresh-cut apples, melons and pears (Abadias et al., 2014; Alegre et al., 2013a; Iglesias et al., 2018). Those results suggest that CPA-7 does not show an enhanced pectinolytic activity affecting fruit quality.

Soluble solids content (SSC) in unprocessed apples was initially higher in H2 apples than in H1 ones (Table 1). It subsequently remained invariable throughout time in apples from both harvests. Interestingly, samples treated with the antioxidant showed an initial increase in the SSC in contrast with untreated samples regardless of the maturity stage. Higher contents in soluble solids were also observed for AOX- treated samples at the end of MAP storage in apples from the second harvest date. In general, inoculation with CPA-7 did not alter SSC in any of the evaluated conditions. Similarly, apple pH was about the same (4.0 ± 0.2) and remained stable throughout the studied period for all the conditions tested. SSC was not significant altered by CPA-7 when

511 inoculated on fresh-cut melon, apples or pears as observed in previous experiments
512 (Abadias et al., 2014; Alegre et al., 2013a; Iglesias et al., 2018).

513 Titratable acidity was initially lower in CPA-7 - inoculated H2 samples compared to the
514 control while no differences were observed between inoculated and non-inoculated H1
515 apples (Table 1). The opposite effect was observed at the end of storage in the
516 hermetic system. In MAP conditions, this parameter did not show differences in the
517 presence of CPA-7 at the end of storage. For the AOX-treated samples no differences
518 were observed between inoculated and non-inoculated samples regardless of the
519 maturity stage.

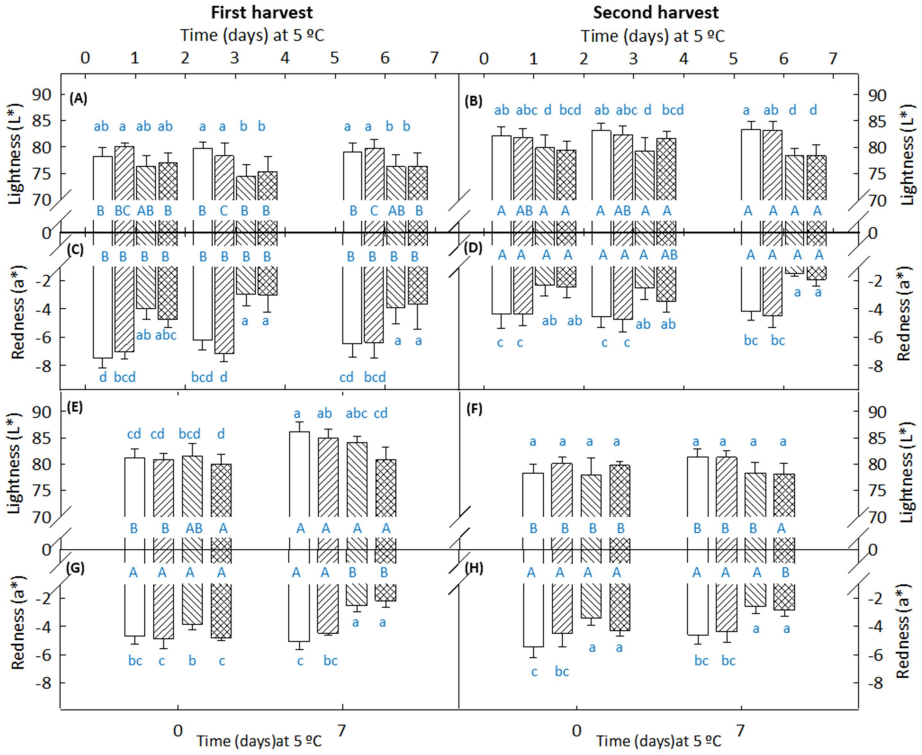
520 Table 1.Chemical quality parameters of intact (Whole) and fresh-cut 'golden delicious' apples from the 1st and the
521 2nd harvest dates, inoculated with CPA-7 (CPA) or non-inoculated (NI), upon antioxidant treatment (AOX) or not
522 (H_2O), when stored in trays (MAP) or in hermetic jars.

Parameter		Jars and trays		Trays		Jars	
		day 0		day 6		day 7	
	Treatment	1st harvest	2nd harvest	1st harvest	2nd harvest	1st harvest	2nd harvest
soluble solids (%)	Whole	12.5 ± 0.1 A	13.3±0.01aA			14±1 aB	13±0.9 aA
	AOX NI	12.8 ±0.01aA	13.6±0.2 aA	12.7±0.04aA	13.3 ±0.03aA	12.3±0.4aA	12.5±0.2 aB
	AOX CPA	12.9 ± 0.1aA	12.8±0.1 bA	12.6±0.1 aA	12.6 ±0.01aA	12.8±0.8aA	13.1±0.2 aA
	H2O NI	12.4 ± 0.1bA	12.4±0.04cA	12.5±0.03aA	11.6 ±0.04bB	12.1±0.2aA	12.4±0.4 aA
	H2O CPA	12.4 ±0.01bA	12.3±0.02cA	12.3±0.2 aA	11.7 ±0.01bB	11.2±0.4aA	12.6±0.1 aA
Titratable acidity malic acid (g L^{-1})	Whole	3.2 ± 0.1 aA	4.4±0.2 aA			2.9±0.4cA	4.6±0.4 aA
	AOX NI	3.3 ± 0.2 aA	3.3±0.1 aA	3.4 ±0.2 aA	3.8 ±0.3 aA	3.8±0.1aB	3.2±0.1 bA
	AOX CPA	3.22 ±0.02 aA	3.37±0.03aA	3.2 ±0.4 aA	3.6 ±0.5 abA	3.8±0.2aB	3.5±0.1 bA
	H2O NI	3.2 ± 0.1aA	2.8±0.5 abA	3.4 ±0.1 aA	3.0 ±0.2 bcA	4.1±0.1aB	3.2±0.1 bB
	H2O CPA	3.4 ±0.1aA	3.1±0.04bA	3.4 ±0.3 aA	3.1 ±0.3 bcA	3.3±0.1bA	3.1±0.04 bA

Results represent mean ± standard deviation. Different lowercase letters represent significant differences among treatments at each sampling time and uppercase letters represent significant differences throughout time for each treatment according to analysis of variances (ANOVA) and Tukey's test ($p < 0.05$)

523 Inoculation with CPA-7 had no influence on a* and L* color parameters compared to
524 non-inoculated control in any of the conditions tested (harvest date, storage system,
525 antioxidant treatment and/or storage period) (Fig. 6). Similar results were obtained after
526 14 d of storage at 5 ºC in a previous study testing the effect of the inoculation with
527 CPA-7 on the quality of fresh-cut apples in semi-commercial conditions resembling the
528 ones assayed in the present work (Alegre et al., 2013a). In general, antioxidant

529 treatment resulted in a reduction of a* after 1 d of MAP storage in inoculated and non-
530 inoculated apples from both harvests (Fig. 6, C-D). However, this effect was more
531 marked in H1 apple wedges than H2 ones. As observed for a*, antioxidant dipping
532 significantly improved and preserved lightness (L*) throughout storage in both storage
533 systems and harvest dates regardless of the inoculation with the antagonist (Fig. 6, A-
534 B, E-F). The effect of AS1 on color maintenance of apple wedges was not that marked
535 in the above mentioned study performed by Alegre et al., (2013a) and could be related
536 to the influence of other parameters related with a different film used for MAP.



537

538 Figure 6. CIE color parameters: L* (lightness) and a* (redness) of fresh-cut apple wedges from the 1st (left column)
539 and the 2nd (right column) harvest dates when stored in trays (MAP): A-D, or in hermetic jars: E-F. Columns
540 represent means of 10 measures per each of three replicate treatments of: non-inoculated antioxidant-treated
541 apples (☐), CPA-7-inoculated antioxidant-treated apples (▨), non-inoculated water-washed apples (▧), CPA-7-
542 inoculated water-washed (▩). Error bars represent standard deviations. Different capital letters represent
543 significant differences among sampling times for each treatment. Different lowercase letters represent significant
544 differences among treatments for each sampling time, according to analysis of variances (ANOVA) and Tukey's test
545 ($p < 0.05$).

546 In general, CPA-7 showed no negative effect on fruit quality parameters when
547 combined with the antioxidant in the tested storage systems. AOX - dipping contributed

548 to maintain physical-chemical fruit quality in the conditions tested in agreement with
549 previous experiments showing that a calcium and ascorbate-containing antioxidant
550 treatment preserved fruit titratable acidity and soluble solids content in fresh-cut 'golden
551 delicious' apples (Soliva-Fortuny, Ricart-Coll, & Martín-Belloso, 2005). However, in
552 agreement with the results obtained by Soliva-Fortuny et al. (2002), we observed that
553 antioxidant dipping was more effective at preserving color parameters in apple wedges
554 from the first harvest than in those from the second one.

555 Conclusions

556 CPA-7 reduced the ethylene production in fresh-cut 'golden delicious' apples after 6 d
557 in MAP which could potentially delay fruit senescence increasing the shelf-life of these
558 kind of products in commercial conditions. However, the inhibition of this effect by the
559 ascorbate-based antioxidant treatment (AS1) limits the possibilities of the use of CPA-
560 7-AS1 combination as hurdle technology. Further studies on the effect of combined
561 strategies including this biocontrol agent and different types of natural chemical
562 preservatives and/or physical methods on the ethylene metabolism of fresh-cut apple,
563 could lead to the advent of better ecofriendly alternatives to improve the feasibility of
564 minimally processed products at a commercial level.

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747 Supplementary table 1. Headspace composition of packages of intact (Whole) and fresh-cut 'golden delicious'
748 apples from the 1st and the 2nd harvest dates, inoculated with CPA-7 (CPA) or non-inoculated (NI), upon
749 antioxidant treatment (AOX) or not (H2O), and when stored in trays (MAP) or in hermetic jars.

			O_2 (%)						CO_2 (%)					
		Treatmen	0.3 d	1 d	2 d	3 d	6 d	7 d	0.3 d	1 d	2 d	3 d	6 d	7 d
Jars (hermetic)	1st harvest	Whole	**20.1** ± 0.1	**17.8** ± 0.0	**17.2** ± 0.4	**16.5** ± 0.4	**15.8** ± 0.1	**15.6** ± 0.1	**0.8** ± 0.1	**2.5** ± 0.1	**3.4** ± 0.3	**4.9** ± 0.2	**6.4** ± 0.2	**6.5** ± 0.4
		NI AOX	**18.3** ± 0.1	**14.9** ± 0.1	**13.6** ± 0.8	**13.4** ± 0.7	**12.3** ± 2	**11.2** ± 0.5	**1.3** ± 0.0	**3.4** ± 0.0	**5.8** ± 0.1	**6.2** ± 0.1	**8.2** ± 1.2	**8.6** ± 0.4
		CPA AOX	**18.4** ± 0.3	**19.6** ± 0.2	**15** ± 2	**15** ± 2	**15.6** ± 2	**14.3** ± 3	**1.1** ± 0.2	**0.4** ± 0.0	**4.0** ± 1	**4.9** ± 2	**5.4** ± 2	**5.8** ± 3
		NI H2O	**19.4** ± 0.2	**15.9** ± 0.3	**13.9** ± 0.3	**13.0** ± 0.6	**9.6** ± 1	**8.9** ± 1	**1.5** ± 0.2	**4.1** ± 0.1	**5.9** ± 0.5	**7.2** ± 0.5	**10.4** ± 0.7	**10.3** ± 0.6
		CPA H2O	**19.0** ± 0.0	**16.1** ± 0.4	**15.2** ± 0.6	**14.2** ± 0.8	**13.0** ± 1	**11.9** ± 0.9	**1.6** ± 0.1	**3.8** ± 0.2	**5.3** ± 0.3	**6.2** ± 0.6	**8.0** ± 1	**8.2** ± 0.7
	2nd harvest	Whole	**18.7** ± 0.1	**15.9** ± 0.4	**13.4** ± 0.8	**11.1** ± 0.4	**7.6** ± 0.9	**6.6** ± 1	**1.5** ± 0.1	**3.9** ± 0.2	**5.7** 0.1	**7.7** ± 0.3	**11.0** ± 1	**12** ± 2
		NI AOX	**18.5** ± 0.0	**14.0** ± 0.5	**10.8** ± 1	**7.3** ± 0.4	**3.9** ± 1	**1.2** ± 0.2	**1.2** ± 0.0	**3.9** ± 0.3	**6.1** 0.7	**6.7** ± 0.7	**12.6** ± 0.6	**14** ± 2
		CPA AOX	**18.3** ± 0.1	**14.6** ± 0.5	**12.3** ± 2	**10.3** ± 0.2	**2.9** ± 0.3	**1.7** ± 0.0	**1.2** ± 0.1	**3.5** ± 0.2	**5.2** 0.4	**7.5** ± 0.6	**12.0** ± 0.8	**13.7** ± 0.2
		NI H2O	**18.3** ± 0.1	**15.3** ± 0.1	**12.9** ± 0.3	**9.1** ± 0.4	**5.8** ± 0.7	**4.2** ± 0.7	**2.0** ± 0.1	**4.5** ± 0.1	**6.1** 0.3	**9.4** ± 0.2	**11.7** ± 0.3	**12.5** ± 0.3
		CPA H2O	**18.6** ± 0.1	**14.7** ± 0.5	**11.7** ± 0.9	**8.3** ± 0.2	**1.2** ± 0.2	**0.4** ± 0.3	**1.9** ± 0.2	**5.3** ± 0.4	**7.3** 0.6	**9.3** ± 0.2	**15.5** ± 0.1	**17.6** ± 0.0
Trays (MAP)	1st harvest	NI AOX		**19.2** ± 0.0	**16.9** ± 0.2	**16.0** ± 0.5	**16.3** ± 0.6	**15.6** ± 0.1		**2.0** ± 0.1	**3.1** ± 0.1	**4.1** ± 0.2	**4.9** ± 0.8	**5.9** ± 0.0
		CPA AOX		**18.6** ± 0.3	**17.7** ± 0.3	**17.5** ± 0.5	**15.3** ± 0.8	**17.2** ± 0.3		**1.5** ± 0.1	**2.7** ± 0.1	**3.2** ± 0.4	**5.5** ± 0.5	**5.1** ± 0.5
		NI H2O		**19.2** ± 0.0	**17.3** ± 0.2	**16.5** ± 0.3	**15.9** ± 0.6	**15.0** ± 0.6		**2.0** ± 0.1	**3.8** ± 0.3	**4.4** ± 0.3	**5.3** ± 1.0	**5.7** ± 0.3
		CPA H2O		**18.9** ± 0.1	**16.3** ± 0.3	**16.0** ± 0.4	**14.8** ± 0.5	**14.8** ± 0.8		**2.3** ± 0.1	**4.2** ± 0.3	**4.9** ± 0.3	**6.4** ± 0.3	**6.0** ± 0.6
	2nd harvest	NI AOX		**17.4** ± 0.6	**16.3** ± 0.7	**13.6** ± 0.5	**14** ± 1	**15.4** ± 0.8		**2.4** ± 0.3	**3.5** ± 0.5	**6.1** ± 0.3	**6.2** ± 0.9	**5.6** ± 0.6
		CPA AOX		**17.3** ± 0.2	**16.0** ± 0.2	**12.5** ± 0.8	**13.4** ± 0.1	**14.5** ± 0.1		**2.4** ± 0.2	**3.7** ± 0.1	**7.1** ± 0.6	**7.5** ± 0.1	**6.8** ± 0.1
		NI H2O		**17.9** ± 0.4	**17.1** ± 1	**15.4** ± 0.6	**14.7** ± 0.8	**16** ± 2		**2.9** ± 0.4	**3.6** ± 0.8	**5.1** ± 0.4	**5.1** ± 0.8	**5** ± 2
		CPA H2O		**17.8** ± 0.1	**16.7** ± 0.0	**14.2** ± 0.8	**15.8** ± 0.6	**16.1** ± 0.4		**2.9** ± 0.1	**4.0** ± 0.1	**5.9** ± 0.7	**5.4** ± 0.7	**5.0** ± 0.6

Initial concentrations were 21 % O_2 and 0 % CO_2. NI: non-inoculated, CPA: CPA-7-inoculated, AOX: AOX-treated, H2O: AOX-untreated. Results are expresed as mean ± standard deviation.

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